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17 MAY 2004
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To:

MALAYSIAN RUBBER BOARD (MRB)
17 & 18 FLOOR, BANGUNAN GETAH ASLI,
148, JALAN AMPANG,
50716 KUALA LUMPUR,
MALAYSIA.

PATENT APPLICATION NO: PI 2003 1807

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ROZANAH MISKAN
(CERTIFYING OFFICER)
12 Mei 2004
rozanah@mipc.gov.my
Tel: 03-22748970

Puan Nurul,
Sila update, kantor sahkan
kepada Dr. Yeong ? fail. Thx
17/5

Perbadanan Harta Intelek Malaysia
(Intellectual Property Corporation of Malaysia)
Aras 32, Menara Dayabumi,
Jalan Sultan Hishamuddin,
50623 Kuala Lumpur

Telefon: 03-2274 8671
Fax : 03-2274 1332
http://www.mipc.gov.my

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CERTIFICATE OF FILING

APPLICANT	:	i) MALAYSIAN RUBBER BOARD (MRB) ii) CENTRE DE COOPERATION INTERNATIONALE EN RECHERCHE AGRONOMIQUE POUR LE DEVELOPMENT (CIRAD)
APPLICATION NO	:	PI 20031807 ✓
REQUEST RECEIVED ON	:	16/05/2003 ✓
FILING DATE	:	16/05/2003
AGENT'S/APPLICANT'S FILE REF.	:	-

Please find attached, a copy of the Request Form relating to the above application, with the filing date and application number marked thereon in accordance with Regulation 25(1).

Date : 27/05/2003

(ROZILEE BIN ASID)
for Registrar of Patents

To : DR. YEANG HOONG YEET
17 & 18 FLOOR,
BANGUNAN GETAH ASLI
148 JALAN AMPANG
50716-KUALA LUMPUR
MALAYSIA

FF 09

Patents Form No. 1
PATENTS ACT 1983

REQUEST FOR GRANT OF PATENT
(Regulation 7(1))

To: The Registrar of Patents
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Kuala Lumpur
Malaysia

For Official Use

APPLICATION NO.: PI 20031807

APPLICATION RECEIVED ON: 16-05-2003

Fee received on: 16-05-2003

Amount: RM200.

*~~Cheque/Postal Order/Money Order/Draft~~/Cash No.: CPFH

Please submit this Form in duplicate together with the prescribed fee.

Applicant's file reference

THE APPLICANT(S) REQUEST(S) THE GRANT OF A PATENT IN RESPECT OF THE FOLLOWING PARTICULARS:

I. TITLE OF INVENTION : Promoter Sequences from Hevea brasiliensis hevein genes

II. APPLICANT(S) (the data concerning each applicant must appear in this box or, if the space is insufficient, in the space below)

Name : (1) Malaysian Rubber Board (MRB) ; (2) Centre De Cooperation Internationale En Recherche Agronomique Pour Le Developpement (CIRAD)

Hsc No./I.C./Passport No. : -

Address : (1) 17 & 18 Floor, Bangunan Getah Asli, 148 Jalan Ampang, 50716 Kuala Lumpur, Malaysia.
(2) 42, rue Scheffer, 75116 Paris, France.

Address for service in Malaysia: 17 & 18 Floor, Bangunan Getah Asli, 148 Jalan Ampang, 50716 Kuala Lumpur, Malaysia.

Nationality : -

*Permanent residence or principal place of business:

as above

DITERIMA 16 MAY 2003 Perbadanan Harta Intelek Malaysia

Telephone Number (if any)	Fax Number (if any)
(1) 03-92062000 (KL) 03-61561121 (Sg. Buloh)	(1) 03-21613139 (KL) 03-61565251 (Sg. Buloh)
(2) 33-467615898 (Paris)	(2) 33-467615659 (Paris)

Additional Information (if any)

PI 20031807

III. INVENTOR

Applicant is the inventor Yes [ ] No [✓]

If the applicant is not the inventor :

Name of inventor : (1) Dr. Arokiaraj Pappusamy ; (2) Dr. Valerie Pujade-Renaud ; (3) Dr. Heddwyn Jones

Address of inventor (1) MRB Experimental Station, Unit Biotechnology & Strategic Research, 47000 Sg. Buloh, Selangor Darul Ehsan, Malaysia.
(2) CIRAD, TA80/03, Avenue Agropolis, 34398 Montpellier Cedex 5, France.
(3) Department of Biosciences, University of Hertfordshire, College Lane, Hatfield, Herts AL10 9AB, United Kingdom.

A statement justifying the applicant's right to the patent accompanies this Form:

Yes [✓] No [ ]

Additional Information (if any)

IV. AGENT OR REPRESENTATIVE

Applicant has appointed a patent agent in accompanying Form No. 17 Yes [ ]

No [✓]

Agent's Registration No. : .....

Applicants have appointed ..... to be their common representative

V. DIVISIONAL APPLICATION

This application is a divisional application [ ]

The benefit of the

filing date [ ] priority date [ ]

of the initial application is claimed in as much as the subject-matter of the present application is contained in the initial application identified below:

Initial Application No. : .....

Date of filing of initial application : .....

VI. DISCLOSURES TO BE DISREGARDED FOR PRIOR ART PURPOSES

Additional information is contained in supplemental box:

(a) Disclosure was due to acts of applicant or his predecessor in title ☐

Date of disclosure : .....

(b) Disclosure was due to abuse of rights of applicant or his predecessor in title ☐

Date of disclosure : .....

A statement specifying in more detail the facts concerning the disclosure accompanies this Form

Yes ☐

No ☐

Additional Information (if any)

VII. PRIORITY CLAIM (if any)

The priority of an earlier application is claimed as follows:

Country (if the earlier application is a regional or international application, indicate the office with which it is filed):

.....

Filing Date: .....

Application No.: .....

Symbol of the International Patent Classification:

If not yet allocated, please tick ☐

The priority of more than one earlier application is claimed:

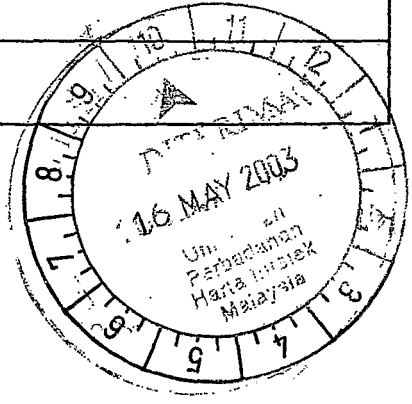
Yes ☐ No ☐

The certified copy of the earlier application(s) accompanies this Form:

Yes ☐ No ☐

If No, it will be furnished by..... (date)

Additional Information (if any)



PI 20031807

VIII. CHECK LIST

A. This application contains the following:

1. request
2. description ..... 22 ..... sheets
3. claim ..... 2 ..... sheets
4. abstract ..... 1 ..... sheets
5. drawings ..... 11 ..... sheets

Total ..... 36 ..... sheets

B. This Form, as filed, is accompanied by the items checked below:

(a) signed Form No. 17	
(b) declaration that inventor does not wish to be named in the patent	
(c) statement justifying applicant's right to the patent	✓
(d) statement that certain disclosures be disregarded	
(e) priority document (certified copy of earlier application)	
(f) cash, cheque, money order, banker's draft or postal order for the payment of application fee	✓
(g) other documents (specify)	

IX. SIGNATURE ..... 16 May 2003

(DR. YEANG HOONG YEET) *(Date)*

DR. H.Y. YEANG

**(Applicant/Agent)

Head of Unit

Biotechnology & Strategic Research

Malaysian Rubber Board

If Agent, indicate Agent's Registration No. : .....

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1. Date application received: .....

2. Date of receipt of correction, later filed papers or drawings completing the application:

.....

* Delete whichever does not apply.

** Type name under signature and delete whichever does not apply.

PROMOTER SEQUENCES FROM *HEVEA BRASILIENSIS* HEVEIN GENES

Technical Field

The present invention relates to the isolation of DNA sequences from the *Hevea brasiliensis* containing the promoter and regulatory region of hevein genes, and to demonstrate their functionality in *Hevea* as well as in heterologous systems. The present invention also provides a recombinant expression cassette comprising the hevein promoter and a heterologous polynucleotide placed under transcriptional control of said promoter. The present invention further provides recombinant expression vectors comprising an expression cassette of the invention, introduced into host cells and plants to produce transformed cells and transgenic plants. For that purpose, chimaeric gene constructs containing hevein 5' flanking DNA linked to the *uidA* reporter gene was prepared and introduced into *Hevea* callus, and for some of the constructs, in model plants (rice or *Arabidopsis*). Functional analysis conducted by histochemical analysis and by measuring the β-glucuronidase enzyme activity, in various contexts revealed the functionality of the hevein promoter in transformed cells and transgenic plants. The promoter sequences of the hevein genes also acts as an inducible promoter regulated by wounds and pathogen infection.

Background of the invention

Hevea brasiliensis (Willd. Ex Adr. Juss) is a tropical perennial euphorbiaceae originating from the Amazon. It is from far the main crop exploited for the production of natural rubber in the world. In *Hevea brasiliensis,* natural rubber occurs as a suspension of cis-1,4polyisoprene particles (from 0.01 to 15 µm in size) surrounded by a single membrane, in the latex, which is the cytoplasm of specialized cells called laticifers. Genetic engineering of *Hevea brasiliensis* may be one way to improve the yield of natural rubber production, by over-expressing favorable genes of the latex metabolism or by improving the defenses of the tree. It is also an interesting tool for the production of recombinant proteins of industrial interest (YEANG et *al.*,

Engineering Crop Plants for Industrial End Uses, (Shewry,P., Napier,J., Davis,P. eds), pp.5563, Portland Press Proceedings, 1998; AROKIARAJ, Molecular Biology of Woody Plants, volume 2 (Mohan,S.J. and Minocha,S.C. eds), pp.305-325, Kluwer Academic Publishers, The Netherlands, 2000). An *Agrobacterium tumefaciens-mediated* transformation procedure of the *Hevea brasiliensis* cultivar Gl 1 has been established (AROKIARAJ et *al.*, Plant Cell Rep. 17, 621-625, 1998) and other procedures are being set up, on other, high yielding, cultivars (MONTORO et *al.*, Plant Cell Reports 19, 851-855, 2000; RATTANA et *al.*, Thai Journal of Agricultural Science 34(3), 2001). Genetic engineering programs require the use of efficient promoters, well adapted to the plant and the application aimed, in order to optimize the expression of the transgene at the right place and the right moment.

The latex vessels, formed by reticulated chains of contiguous anastomosed cells, are periodically emitted from the cambium towards the phloem, as concentric rings. Upon tapping (excision of a thin layer of the trunk bark about 1 mm thick), the latex vessels are severed and the latex flows out, until latex coagulation processes occur to plug the wound. Upon centrifugation at high speed, the expelled latex separates into three fractions: the rubber fraction, the C(cytosol)-serum and the "bottom" fraction composed of sedimentable organelles, mainly vacuolar elements known as lutoids. The B-serum, fluid found insides the lutoids, is very rich in proteins. One of the major proteins in the B-serum is hevein (ARCHER et *al.*, Rubber Res. Inst. Malaya 21, 560-569, 1969), that represents 50-70% of the B-serum soluble proteins and about 2 to 2.8 mg per ml of total latex. Hevein is a small single chain protein of 43 amino acids, unusually rich in cysteine and glycine (WALUJONO et *al.*, Proc. Internat. Rubber Conf., Kuala Lumpur, pp 518-531, 1975). It is a monomer and has an apparent molecular weight of 9.5 kDa as determined by gel filtration and SDS-PAGE (VAN PARIJS et *al.*, Planta 183, 258-264, 1991). It is a chitin-binding protein that participates in the latex coagulation processes when released in the cytosol, by fixing the N-acetyl-D-glucosamine moiety of a receptor protein

located at the surface of the rubber particles, therefore promoting their agglutination and the plugging of the tapping cut (GIDROL et *al.*, J. Biol. Chem. 269, 9278-9283, 1994).

The mature hevein originates from a 204 aminoacids precursor protein which is matured by co- and post-translational processing, giving rise to 2 distinct domains (BROEKAERT et *al.*, Proc. Natl. Acad. Sci. U.S.A. 87, 7633-7637, 1990; LEE et *al.*, J. Biol. Chem. 266(24), 15944-15948, 1991): the 43 aminoacids N-terminal chitin-binding domain (hevein), specific of the lectins superfamily (VAN DAMME et *al.*, Plant Physiol. 119, 1547-1556, 1999) and a 144 aminoacids C-terminal domain, homologous to the wound-inducible proteins WIN1 and WIN2 from potato (STANFORD et *al.*, Mol. Gen. Genet. 215, 200-208, 1989) and to type IV Pathogenesis-related proteins (PR-IV) isolated from tobacco and tomato (FRIEDRICH et *al.*, Mol. Gen. Genet. 230, 113-119, 1991; LINTHORST et *al.*, Mol. Plant Microbe Interact. 4, 586-592, 1991; VAN DAMME et *al.*, 1999, cited above). In *Hevea*, prohevein can thus be classified as a PR-IV protein of class I, characterized by the presence of the chitin-binding N-terminal domain (VAN DAMME et *al.*, 1999, cited above). In addition to these sequence homologies with PR proteins, other evidences suggest that hevein plays a role in defense. The hevein capacity to inhibit fungal growth was demonstrated *in vitro* (VAN PARIJS et *al.*, 1991, cited above). In transgenic tomato plants, hevein, although poorly cleaved, displayed effective antifungal properties (LEE and RAIKHEL, Brazilian Journal of Medical & Biological Research 28, 743-750, 1995).

Additionally, in *Hevea*, hevein is expressed at a high level in the latex compared to leaves, and is over-expressed by wounding, ethylene, and ABA (BROEKAERT et *al.*, Proc. Natl. Acad. Sci. U.S.A. 87, 7633-7637, 1990).

In conclusion, hevein is proposed to participate in the protection of the *Hevea* trunk tissues, recurrently severed by the tapping process, by allowing the sealing of the tapping cut through the latex coagulation process and by preventing pathogenic infection.

Only one nucleotide sequence encoding the full hevein precursor protein had been described so far: GenBank M36986 (BROEKAERT et *al.*, 1990, cited above).

The 5′ upstream region including the putative promoter sequences of two hevein genes has been released: GenBank AF327518 (DENG et *al.*); GenBank AF287016 (AROKIARAJ and JONES).

However until now, nothing was known about the actual functionality and efficiency of these sequences as promoters for driving the expression of heterologous genes.

The inventors have now shown that the hevein precursors belong to a multigene family, that may be organized in two groups (group I and group II) based on nucleotide sequence homology. Further, the inventors have demonstrated that the 5' upstream region isolated from 3 different hevein genes, namely PHev1.1, representative of the hevein group I, and PHev2.1 (1824 bp) and PHev2.3 (corresponding to AF287016) (966 bp), representatives of the hevein group II, are able to drive the expression of a transgene in *Hevea*. They are therefore functional as promoters. Additionally, the inventors have demonstrated that these promoters are also functional in rice, model plant for the monocots, and *Arabidopsis,* model plant for the dicots. Thus the promoter sequences from the various hevein genes may be used as promoters in transformation programs, not only in *Hevea* but also in various other heterologous systems.

Summary of the Invention

The first aspect of the present invention provides the shortest sequence shown by the inventors to be functional in *Hevea* as well as in rice is the PHev1.1 sequence. This sequence thus represents a promoter sequence able to drive the expression of a transgene in various plants, and constitutes a basis for the isolation of a longer promoter sequence, more readily usable as promoter in transformation programs.

The second aspect of the present invention provides the longest regulatory sequence tested by the inventors (PHev2.1) was demonstrated to be up-regulated by mechanical wounding in rice, with the capacity to respond systemically. It was also upregulated by pathogen infection.

Further, the present invention provides the use of a polynucleotide selected among:

(a) a polynucleotide having a sequence including nucleotides -254 to -1 of the sequence of Figure 3 (nucleotides 1-254 of SEQ ID NO: 1);

(b) a polynucleotide having a sequence including nucleotides -448 to -1 of the sequence represented of Figure 4 (nucleotides 1-448 of SEQ ID NO: 2);

(c) a polynucleotide having a sequence including nucleotides -1776 to -1 of the sequence of Figure 5, (nucleotides 1-1776 of SEQ ID NO: 3);

(d) a polynucleotide having a sequence including nucleotides -1044 to -1 of the sequence of Figure 6, (nucleotides 1-1044 of SEQ ID NO: 4);

(e) a polynucleotide having a sequence including nucleotides -919 to -1 of the sequence of Figure 7, (nucleotides 1-919 of SEQ ID NO: 5;

(f) a polynucleotide consisting of a fragment of at least 254 bp from the 3'end of a polynucleotide (b) to (e);

(g) a polynucleotide having at least 90% identity with a polynucleotide (a) to (f) and having a promoter function in a plant cell; as a promoter for driving the expression of an heterologous gene of interest in a plant. Advantageously, the polynucleotide (g) is selected among:
a polynucleotide having at least 95% identity with any of the polynucleotides (a) or (b);
a polynucleotide having at least 90% identity with any of the polynucléotides (c) to (f).

According to a preferred embodiment of the invention, one will use a polynucleotide selected among;

(a') a polynucleotide of SEQ ID NO: 1;

(b') a polynucleotide of SEQ ID NO: 2;

(c') a polynucleotide of SEQ ID NO: 3;

(d') a polynucleotide of SEQ ID NO: 4;

(e') a polynucleotide of SEQ ID NO: 5;

(f') a polynucleotide consisting of a fragment of at least 308 bp from the 3'end of a polynucleotide (b') to (e');

(g') a polynucleotide having at least 90% identity with a polynucleotide (a') to (f') and having a promoter function in a plant cell.

Preferably, said polynucleotide (g') comprises a sequence having at least 95% identity with a 97 bp sequence immediately upstream the ATG of a polynucleotide (a) to (f).

According to a preferred embodiment of the invention, a polynucleotide selected among:

- a polynucleotide (c) or (c')as defined above;
- a polynucleotide (g) or (g')having at least 90% identity with said polynucleotide (c) is used as an inducible promoter regulated by wounds and pathogen infection.

The present invention also provides a recombinant expression cassette comprising a promoter consisting of a polynucleotide (a) to (g), or preferably a polynucleotide (a') to (g') as defined above, and a heterologous polynucleotide placed under transcriptional control of said promoter. The term "heterologous polynucleotide" refers herein to any polynucleotide other than an hevein coding sequence. Said heterologous polynucleotide may consist for instance of a coding or of an antisense sequence of interest operably linked to said promoter, or of a cloning site for inserting said sequence of interest.

The present invention further provides recombinant expression vectors comprising an expression cassette of the invention. These expression vectors can be introduced into host cells and plants to produce transformed cells and transgenic plants, using methods

known in the art. Said transformed cells, in particular plant cells, and transgenic plants are also part of the invention.

The present invention is suitable for use not only in *Hevea* and other plants of the euphorbeaceae family such as cassava or castor bean, but also in other dicotyledonous plants (as shown by the functionality of promoter sequences of the invention in the model plant *Arabidopsis*), including in particular plants producing latex such as guayule, sunflower, lettuce, dendelion or papaya.

Further, the present invention is also suitable for use in monocotyledonous plants, including in particular cereals, such as rice, maize, wheat, barley, or oats, and also including, in a non-limitative way, other plants such as banana, sugar cane or palm trees.

Brief Description of Figures

Figure 1A shows the synthetic oligonucleotide primers, starting with the primers T3, T7 and the reverse primer OR1, designed from the hevein cDNA sequence (GenBank accession M36986, position 58 to 83) for the isolation of the hevein gene promoter region PHev1.1, PHev1.2, PHev2.1 and PHev2.2

Figure 1B shows the sequences of all the oligonucleotide primers used for the isolation and cloning of HevP (PHev2.3)

Figure 2 shows the classification of the hevein genes and their subclones. Cloning I: genomic sequences including the full transcribed region and 5′ upstream sequences of hevein genes, cloned in the pBlueScript phagemid from lambda Zap II (stratagene). Cloning II: Promoter regions isolated by PCR for Hev 1.1, Hev 1.2, Hev2.1 and Hev2.2, subcloning from the pBS-Hev clones into the pGEMT-easy vector (Promega) and Hev2.3 (HevP), direct cloning by adaptor-anchored PCR in the pCR2.1 TOPO vector (Invitrogen, USA).

Cloning III: Subcloning in a binary vector for transformation. For Hev2.1 and Hev2.2, isolated promoter region cloned between the Hind *III* and Bgl *II* sites of the pCambia1381Z vector (Cambia). For Hev2.3 (HevP), deleted fragments of the isolated promoter region cloned between the Xba *I* and Hind *III* sites of the pPGTV-KAN vector.

Figure 3 shows the nucleotide sequence of the hevein promoter region Phev1.1. The transcription start site (A) and the translation initiation codon (ATG) are in bold letters.

Figure 4 shows the nucleotide sequence of the hevein promoter region Phev1.2. The transcription start site (A) and the translation initiation codon (ATG) are in bold letters.

Figure 5 shows the nucleotide sequence of the hevein promoter region Phev2.1. The transcription start site (A) and the translation initiation codon (ATG) are in bold letters.

Figure 6 shows the nucleotide sequence of the hevein promoter region Phev2.2. The transcription start site (A) and the translation initiation codon (ATG) are in bold letters.

Figure 7 shows the nucleotide sequence of the hevein promoter region Phev2.3 (HevP), isolated by nested PCR. The transcription start site (A) and the translation initiation codon (ATG) are in bold letters.

Figure 8 shows the schematic diagram of the PCR-generated nested deletion fragments obtained from the hevein promoter region HevP (PHev2.3) and cloned in pPGTV-KAN. The promoter sequences were joined to the *uidA* reporter gene. The 5′ and 3′ end points of the promoter sequences are numbered from the transcription start site of the hevein gene.

Figure 9 shows the fluorometric analysis of the GUS activity in transgenic rice plants (T1) carrying a single copy of the *uidA* gene driven by PHev2.1, in response to mechanical wounding.

Figure 10 shows the results of fluorometric analysis of the GUS activity I transgenic rice plants (T1) carrying a single copy of the *uidA* gene driven by PHev2.1, in response to fungal infection.

Detailed Description of Invention

By way of example, the present invention provides interesting tools for the genetic engineering of *Hevea* with the purpose of improving existing genotypes for natural rubber production, or for molecular pharming.

For instance, in *Hevea* genetic engineering programs, the use of a wound-inducible promoter will guaranty a high transgene expression level in the exploited bark tissues, owing to the regular wound stress imposed by tapping. This promoter will also be useful for increasing the expression of endogenous genes identified as limiting factors for the yield of latex production. The invention also allows to optimize transgene expression in the latex cells; this is of particular importance for programs of molecular pharming aiming at producing exogenous molecules of industrial interest in the latex.

In a more general way, the present invention can be used in *Hevea* as well as in other plant systems, for improving the defenses of plants against biotic and abiotic stresses. The use of a promoter inducible by wounds and pathogen infection allows to over-express transgenes involved in defense, in situations such as aggressions by plant-eater insects or infection by microorganisms such as fungi.

The present invention will be further illustrated by the additional description which follows, which refers to the isolation of the 5′ upstream regions or several hevein genes and their use for expressing heterologous DNA in different plants. It should be understood however that these examples are given only by

way of illustration of the invention and do not constitute in any way a limitation thereof.

EXAMPLE 1: CLONING OF HEVEIN-ENCODING GENOMIC SEQUENCES AND ISOLATION OF THE 5′ UPSTREAM REGION

In *Hevea*, hevein is encoded by a small multigene family. We have isolated 5 different genomic sequences corresponding to hevein genes. Four sequences (Hev1.1, Hev1.2, Hev2.1 and Hev2.2) are including the full coding sequence of the hevein precursor as well as the 5′ upstream region. One sequence includes the partial coding sequence and 5′ upstream region of a fifth hevein gene (Hev2.3). A partial sequence of Hev2.3 is available in GenBank under the accession AF287016.

Cloning of Hev1.1, Hev1.2, Hev2.1 and Hev2.2 by library screening and isolation of the 5′ upstream region

Genomic library construction and screening:

Genomic DNA was extracted from young leaves of Hevea brasiliensis cultivar RRIM600, using the method described by DELLAPORTA et *al.* (Plant genetic Transformation and Gene Expression. A laboratory Manual, (Draper,J., Scott,R., Armirage,P., Walden,R. eds), pp.214-216, Blackwell Scientific, London, UK, 1985).

A genomic library was constructed by ligating *Eco*R I-digested genomic DNA to the *Eco*R I site of the Lambda Zap II vector (Stratagene). Phages were packaged using the Stratagene "Gigapack III Gold Packaging extract" and plated in the host strain XL1-blue MRF', as described by the manufacturer. Two rounds of screening were performed using a 1 kb cDNA probe corresponding to the full length hevein cDNA (BROKAERT et *al.*, 1990, cited above). Hybridization was performed overnight at 65°C, in 5 x SSC (0.3 M NaCl, 30 mM trisodium-citrate, pH 7), 10x Denhardt's reagent (0.2% ficoll, 0.2% PVP, 0.2% BSA), 7% SDS, 20 mM sodium phosphate buffer pH 7.2 and 100 µg ml^{-1} denatured salmon sperm DNA. Final washes were carried out at 65°C, in 0.1 x SSC and 0.5% SDS. From the

selected phage colonies, the pBlue-Script SK(-) phagemids containing the cloned genomic inserts were excised by co-infection with the ExAssist helper phage and transferred into *E. coli* XL1 blue. The excised phagemids were extracted using Quiagen kits and mapping was performed using the enzymes *Kpn*I, *Eco*RI, *Sac*I, *Hind*III, *Pst*I and *Xho*I, in TA buffer (33 mM Tris acetate pH 7.9, 60 mM K^+ acetate, 10 mM Mg^{2+} acetate, 0.5 mM DTT and 0.1 mg/ml of BSA, added extemporarily), at 37°C. The restriction fragments, separated by electrophoresis in 0.8% agarose gel, were transferred onto nylon membranes and analyzed by Southern hybridization, to determine the orientation of the hevein gene inside the cloning vector. Two probes were used successively: a cDNA fragment located near the 5' terminus of the cDNA (GenBank accession M36986, position 1-91), and the last 339 bp of the cDNA, including the whole 3' non coding region.

Four clones differing in restriction map were selected and sequenced on both strands using synthetic oligonucleotide primers, starting with the primers T3, T7 and the reverse primer OR1, designed from the hevein cDNA sequence (GenBank accession M36986, position 58 to 83). These primers are shown in Figure 1A.

Then new primers were designed successively from each new sequence obtained. The four clones were named pBS-Hev1.1, pBS-Hev1.2, pBS-Hev2.1 and pBS-Hev2.2.

Subcloning I:

Phagemids pBS-Hev1.1 and pBS-Hev2.1, were chosen for PCR amplification of the hevein gene 5' upstream region. The forward primer SCH-S2 (Fig. 1A) was designed from the pBlue-Script SK phagemid vector, 12 bp from the T3 promoter. The reverse primer SCH-R2 (Fig. 1A) was designed from the hevein gene upstream sequence PHev2.1, at position +27 to +48 from the transcription start site, 6 bp upstream the ATG described by BROEKAERT et al. (1990, cited above) as the translation initiation codon. Amplification was performed in a Perkin Elmer thermocycler, in a 50 µl final volume, using 2 ng of *Kpn*I-linearized phagemid as matrix, and DNA polymerase mix and buffer from the "Expand High Fidelity PCR System" (Boehringer), supplemented with $MgCl_2$ (3 mM).

Amplification was performed over 35 cycles under the following conditions: denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec and elongation at 72°C for 2 min. The PCR fragments were purified by electrophoresis in 1.2% low melting agarose gel and eluted on affinity columns (NUCLEOSPIN Extract, Macherey-Nagel) and ligated in pGEM-T *easy* vector (Promega).

The new constructs, named pGEMT-PHev1.1 and pGEMT-PHev2.1 (Fig. 2) were introduced into E *Coli* DH5α bacteria by electroporation.

Subcloning II:

The plasmid pCAMBIA 13812 (CAMBIA) was restricted using the enzymes *Hind*III and *Bgl II* in buffer II (Boehringer Mannheim), then dephosphorylated using alkaline phosphatase AP (Boehringer Mannheim).

The plasmids pGEMT-PHev1.1 and pGEMT-PHev2.1 were restricted using the enzymes *Hind*III and *Bgl*II in buffer II (Boehringer Mannheim). The fragments corresponding to the promoter regions were isolated on 1.2% low melting agarose gel and purified using NUCLEOSPIN Extract columns (Macherey-Nagel). They were ligated into the *Hind*III/*Bgl*II-restricted and dephosphorylated vector pCAMBIA 13812. The new constructs, named pCAMBIA-PHev1.1 and pCAMBIA-PHev2.1 (Fig. 2), were introduced into *Agrobacteria* (strain LBA 4404) by electroporation. The constructs were re-extracted from the *Agrobacterium* using QIAGEN plasmid extraction system and checked by restriction profile analysis using the enzymes *XhoI* and *Eco* RV and by sequencing.

The four clones isolated (Hev1.1, Hev1.2, Hev2.1 and Hev2.2), include the full transcribed region of four different hevein genes, together with about 300 to 1.8 kb of their 5' upstream sequence.

They were classified in 2 groups based on nucleotide sequence homology. The members of group I (Hev 1.1 and Hev 1.2) share about 96% identity while the members of group II (Hev2.1 and Hev2.2) share 99% identity. The percentage of identity between members of the two different groups is about 86%.

Cloning of HevP (PHev2.3) by adaptor-anchored PCR and subcloning.

A fifth clone (HevP, or PHev2.3) including the leader peptide and 5' flanking region of another hevein gene (Hev2.3) belonging to the hevein group II gene family, was isolated by adaptor-anchored PCR.

High-molecular weight DNA was isolated from young leaves of RRIM 600 *(Hevea brasiliensis)* using the method as described by DELLAPORTA et *al.* (1985, cited above). Genomic mini-libraries were constructed using the *Hevea* genomic DNA by using the Universal GenomeWalker™ Kit according to manufacturer's instructions (Clontech Laboratories, Inc, CA, USA). The mini-libraries were used as template DNA for PCR and nested PCR reactions for the isolation of hevein upstream sequences. The oligonucleotide primers were synthesized by Operon (Operon Technologies Inc, USA). The sequences of all the oligonucleotide primers used are listed in Figure 1B.

Primary PCR reaction:

DNA amplification was carried out on a thermal cycler, in a 50 µl volume, containing 5 µl of 10x Tth PCR reaction buffer (Clontech), 2.2 µl of 25mM $Mg(OAc)_2$, 1 µl of 10 µM of each primer (GSP3 hevein and AP1), 1 µl of 10 mM each dNTPs, 1 µl of Advantage Polymerase Mix (2.5 units) (Clontech), 1 µl of the genomic mini libraries and 37.8 µl of deionised H_2O. Using a two-step cycle parameter in a DNA Thermal Cycler 480 (PE Biosystems) the PCR reaction was carried out as follows: 7 cycles at 94°C for 25 sec and 72°C for 3 min; 32 cycles at 94°C for 25 sec and 67°C for 3 min; 67°C for an additional 7 min after the final cycle. Eight microliters of the primary PCR product were analyzed on a 1.5% agarose gel along with DNA size marker (Gibco BRL 1 kb Plus Ladder).

Secondary PCR reaction:

A secondary PCR reaction was performed using a 1:50 dilution of the primary PCR product. DNA amplification was carried out on a thermal cycler, in a 50 µl volume, containing 5µl of 10x Tth PCR reaction buffer (Clontech), 2.2 µl of 25 mM $Mg(OAc)_2$, 1 µl of 10 µM of each primer (GSP2 hevein and AP2), 1 µl of 10 mM each dNTPs, 1 µl of Advantage Polymerase Mix (2.5 units) (Clontech), 1 µl of

the diluted (1:50) primary PCR product as template DNA and 37.8 µl of deionised H_2O. Using a two-step cycle parameter in a DNA Thermal Cycler 480 (PE Biosystems) the PCR reaction was carried out as follows: 5 cycles at 94°C for 25 sec and 72°C for 3 min; 20 cycles at 94°C for 25 sec and 67°C for 3 min; 67°C for an additional 7 min. after the final cycle.

Five microliters of the secondary PCR products were analyzed on a 1.5% agarose gel, along DNA size marker (Gibco BRL 1 kb Plus Ladder). The amplified product (1.3 kb) was excised from the gel and purified. The purified DNA fragment was cloned into TOPO Cloning TA vector pCR®2.1TOPO® (Invitrogen, USA) and transformed in E. *Coli* DHSα. Plasmid extractions were performed using the Qiagen Plasmid Mini Kit (Qiagen Inc, USA). The 1.3 kb insert was released by restriction using the enzyme *EcoR I*, then sequenced (Strathclyde University, Department of Molecular Biology, United Kingdom) using as primers, M13R and M13F.

Comparison of the promoter sequences of the hevein genes

The transcription start site was identified by primer extension from the clone PHev2.3, and deduced by sequence homology for the others. It is located 54 nt upstream the ATG codon described as the hevein mRNA translation start point.

The 5' upstream sequences of the five hevein genes are presented in Figures 3 (PHev1.1.), 4 (PHev1.2.), 5 (PHev2.1.), 6 (PHev2.2.)and 7(PHev2.3.). The transcription start site (A) and translation initiation codon (ATG) are in bold letters.

The 5' upstream regions of the 5 sequences PHev1.1, PHev1.2, PHev2.1, PHev2.2 and PHev2.3 were aligned together with a sequence released in the GenBank public database under the accession number AF327518.

All six promoter sequences are highly homologous (96% identity) over a 97 bp sequence immediately upstream the ATG (beginning at position -40 from the transcription start site). Further upstream, the sequences diverge in two different groups, with 39-43% identity only between members of the 2 groups. A high sequence homology is conserved among each group, with about 97% identity

shared by members of group I (PHev1.1 and PHev1.2) and 94-98% identity shared by members of group II (PHev2.1, PHev2.2, PHev2.3 and AF327518). PHev2.3, although clearly belonging to group II, lacks a 30 bp domain located at position -173-143 from the transcription start site, when compared to PHev2.1, PHev2.2 and AF327518 .

EXAMPLE 2: FUNCTIONAL ANALYSIS IN *HEVEA*

Deletion fragments from HevP (PHev2.3).

The clone HevP (PHev2.3) was deleted to generate 4 overlapping fragments of decreasing length in the upstream region.

PCR generated fragments of hevein upstream region were derived from the HevP clone using as primers Hev PF, Hev P1, Hev P2, Hev P3 and Hev P4 (Fig. 1B) following routine PCR protocol (HAMILL et *al.*, Plant Cell Rep. 10, 221-224, 1991). The amplified fragments were double digested with *Xba*I/*Hind*III, then purified and ligated to pGPTV-KAN (BECKER et *al.*, Plant Mol. Biol. 20, 1195-1197, 1992) digested with *Xba*I/*Hind*III and dephosphorylated.

The binary vector pGPTV-KAN contains unique cloning sites upstream of the *uidA* gene which allows the insertion of promoter fragments. The T-DNA nopaline synthase (pAnos) and gene 7 (pAg7) poly(A) signals follow the *uidA* gene and the selectable marker *(nptII)* genes, respectively.

In the four constructs generated (pGPTV-KAN-1, pGPTV-KAN-2, pGPTV-KAN-3 and pGPTV-KAN-4), the hevein upstream fragments are fused to the *uidA* gene. The correct orientation and sequence of the fusions were verified.

A schematic diagram of these constructs is represented in Figure 8. The 5' and 3' end points of the promoter sequences are numbered from the transcription start of the Hevein gene. pGPTV-KAN-1(-275 to +55); pGPTV-KAN-2 (-389 to +55); pGPTV-KAN-3 (-687 to +55) and pGTV-KAN-4 (-919 to +55). Arrows indicate direction of transcription; R, right TDNA border; L, left T-DNA border. Abbreviations: St=*Sst*I, *E*=*Eco*RI, Sm=*Sma*I, *X*=*Xba*I, S=*Sal*I,

*H=Hind*III, *B=Bg*II, *Bm=Bam*HI, *npt* II=neomycin phosphotransferase II gene.

The 4 constructs generated (pGPTV-KAN-1; pGPTV-KAN-2; pGPTV-KAN-3; pGPTV-KAN-4), together with pCAMBIA2301 as positive control, were transferred into *Agrobacterium tumefaciens* GV2260 by electroporation (SHEN and BRIAN, Nucleic Acid Research 17, 8395, 1989). The *Hevea brasiliensis* cultivar GL1 was transformed using the *Agrobacterium tumefaciens*-mediated protocol described by AROKIARAJ et *al.* (1998, cited above)

The transformed cells, selected on kanamycin for about 2 months, were then screened for GUS expression by histochemical staining. GUS activity is indicated by a blue coloration after incubation with X-Gluc.

The calli carrying the various hevein promoter constructs and positive control pCAMBIA2301 displayed a blue colored surface, whereas this was not observed in the negative control sample. The same coloration was observed also in embryoids for all constructs except the negative control sample.

These results demonstrate that the various fragments isolated from the hevein PHev2.3 upstream regulatory sequence, from the longest (position -919 to +55) to the shortest (position -276 to +55), are able to drive the transcription of the *uidA* reporter gene in *Hevea* callus and embryoids.

PHev1.1 and PHev2.1

The constructs pCAMBIA-PHev1.1 and pCAMBIA-PHev2.1, containing respectively about 0.3 kb and 1.8 kb of the upstream sequence of Hev1.1 and Hev2.1 (hevein genes representative of group I and II respectively) fused to the *uidA* reporter gene, were introduced into inner integument-derived *Hevea* callus, by microprojectile bombardment-mediated transformation, to verify their functionality.

The cultivar RRIM 600 was transiently transformed by microprojectile bombardment of callus initiated from inner integument and maintained as described by CARRON et *al.* (Biotechnology in Agricultural and Forestry, (Bajaj, Y.P.S. ed),

pp 353-369, Springer Verlag, Berlin Heidelberg, 1995). The particle gun was from BIORAD and the transformation procedure was as described by the manufacturer, with a 1100 psi rupture membrane. The distance between the rupture membrane and the macro-carrier was 6 cm. The distance between the macro-carrier and the callus was 6 cm.

Transient expression analysis *via* histochemical GUS staining revealed spots of blue cells for both constructs, demonstrating that the isolated PHev1.1 and PHev2.1 upstream regulatory sequences are able to drive the expression of the *uidA* reporter gene in *Hevea* callus.

EXAMPLE 3: FUNCTIONAL ANALYSIS IN RICE

The constructs pCAMBIA-PHev1.1 and pCAMBIA-PHev2.1 were introduced in rice, model plant for the monocots, *via Agrobacterium*-mediated transformation. The pCAMBIA-1301 vector, bearing the *uidA* gene fused to the CaMV 35S promoter, was used as control.

The rice cultivar *Nipponbare* (Japonica) was transformed *via* *Agrobacterium tumefaciens* following the procedure described by SALLAUD et *al.* (Theoritical and Applied Genetics, *in press).* Dehulled mature seeds were sterilized and incubated on NB medium in the dark, as described in CHEN et *al.* (Plant Cell Rep. 18, 25-31, 1998), for the production of nodular callus units. Selected units (3-5 mm) were immersed for 10-15 min. in an *Agrobacterium* suspension (OD_{600} of 1) in CCL liquid co-culture medium (R2 medium from OHIRA et *al.*, Plant Cell Physiol. 14, 1113-1121, 1973; supplemented with 2.5 mg/l 2,4-D, 10 g/l glucose, 100 µM acetosyringone, pH 5.2), then blotted dry and transferred onto solid co-culture medium (CCL with 7 g/l agarose). After 3 days at 25°C in the dark, the calli were transferred to R2S selection medium (R2 medium containing 30 g/l glucose, 50 mg/l hygromycin, 400 mg/l cefotaxime, 100 mg/l vancomycine, 7 g/l agarose, pH6.0), at 27°C in the dark. After 2 weeks of selection, the calli were transferred to NBS medium (NB basic supplemented with 2.5 mg/l 2,4-D, 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein

hydrolysate, 50 mg/l hygromycin, 400 mg/l cefotaxime, 100 mg/l vancomycine, 7 g/l agarose, pH6.0). The resistant globular structures developed from the brownishing callus were separated and incubated for 10-15 days on fresh NBS medium, then placed on PRAG pre-regeneration medium (NB basic supplemented with 2 mg/l BAP, 1 mg/l NAA, 5 mg/l ABA, 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein nydrolysate, 50 mg/l hygromycin, 100 mg/l cefotaxime and 100 mg/l vancomycine, 7 g/l agarose, pH5.8), for one week. Creamy_white lobed calli were transferred to RN regeneration medium (NB basic supplemented with 3 mg/l BAP, 0.5 mg/l NAA, 30 g/l glucose, 50 mg/l hygromycin, 4.5 g/l Phytagel, pH5.8), for 2 days in the dark then for 3 weeks with a 12 hours photoperiod. Shoots regenerating from a resistant callus were subcultured in test tubes containing MS medium (MURASHIGE and SKOOG, Physiol. Plant 15, 473-497, 1962) with 50 g/l glucose, 2.6 g/l Phytagel, pH5.8. After 3 weeks, they were transferred to Jiffy peat pellets for 15 days, then to soil pots in the greenhouse.

Only the hygromycin-resistant plants presenting a single T-DNA insertion were selected, by Southern blotting, for further analysis.

Histochemical analyses of β-glucuronidase (GUS) activity were performed as described by JEFFERSON et *al.* (EMBO J. 6(13), 3901-3907, 1987), on : a) basal section of an unwounded leaf; b) section of a leaf wounded with needles; c) root; d) immature seed with pollen bags; e) longitudinal section of an immature seed; f) immature flower; g) pollen grains.

In case of the longest promoter sequence (PHev2.1), blue coloration after incubation with X-Gluc was observed in wounded leaf tissues (cut hedge or needle impacts), for all of the 8 plants analyzed, demonstrating that the PHev2.1 sequence is functional as a promoter and potentially wound-inducible in rice. In roots, blue coloration was observed in the vascular tissue, for all plants tested but in some of the roots only. In the floral tissue, a strong coloration was observed in the pollen bags and in 5-10% of the pollen grains. Patches of coloration were frequently

observed on the lemma and palea, on the seed tegument, and occasionally in the scutellum.

In case of PHev1.1, GUS activity was detected for some of the plants only (5 plants out of 9), in wounded leaf tissues and in root vascular tissues. In the flowers and grains, the coloration was limited to the lemma and palea.

Histochemical analysis of GUS activity in plants of first generation (T0) demonstrates that both constructs are functional in such heterologous system.

Fluorimetrical analyses were performed as described by JEFFERSON et *al.* (1987, cited above). Explants were frozen in liquid nitrogen and stored at -80°C. Total soluble proteins were extracted by grinding 200 mg of explant in 500 µl extraction buffer (50 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 5% v/v glycerol, 1mM DTT, 0.1% v/v Triton). After centrifugation at 20000 g for 15 min at 4°C, the supernatant was collected and centrifuged again for 5 min in the same conditions. Proteins from the supernatant were quantified (Bradford, 1976) using a Multiscan RC (Labsystems) spectrophotometer, coupled to the software Genesis version 2.00 (Labsystems). The fluorescence of 4-MU (4-methylumbelliferone) generated after cleavage of the 4-MUG (4-methylumbelliferyl ß-D-glucuronic acid) substrate by the GUS enzyme, was measured at 460 nm using the fluorimeter "Fluoroscan II version 6.3 (Labsystems)" coupled to the software Genesis version 2.00.

Fluorimetrical analysis revealed that the GUS activity was much higher in plants bearing PHev2.1 than in plants bearing PHev1.1 (with an average value of 243 against 11 pmol MU/min/µg of proteins, in T1 plants), owing probably to the difference in size of the two promoter regions. Moreover, all the PHev2.1-carrying plants tested (8/8) expressed the *uidA* gene, whereas for the PHev1.1-carrying plants, GUS activity was measurable by fluorimetry for 2 plants only, out 9 tested. The GUS activity measured in T1 plants carrying the CaMV 35S promoter was 470 pmol MU/min/µg of proteins in average. Therefore, it appears that the strength of the 1.8 kb PHev2.1 promoter region is about half that of the 35S promoter, in normal conditions.

Assays of the inducibility of PHev1.1 and PHev2.1 in transgenic rice.

T0 transgenic plants presenting a single T-DNA insertion, as determined by Southern blot analysis, were transferred to the green house and grown for seed production (T1 population). The growth conditions were strictly controlled: 25°C, 75% humidity and 11 h 30 min under artificial light (400 µmolE.m-2.s^{-1}). Seeds were collected and dried at 37°C for 3 days, then kept at room temperature. For the selection of hygromycin-resistant T1 lines (transformed plants of second generation), seeds were surface-sterilized and placed in a Petri dish on Whatman paper soaked with 50µg.ml^{-1} hygromycin. After five to seven days, germinated seeds were transferred to the green house.

Tests were performed on 4-5 weeks old seedlings. Each transgenic line was represented by 3 T1 progenies in each condition. Fluorometric analyzes were performed on full leaves, to avoid artifacts linked to the possible existence of a gradient of expression of the hevein promoter along the leaves.

Mechanical Wounding:

The regulation of the hevein promoters by mechanical wounding was verified by fluorimetrical analysis. Mechanical wounding was performed by pricking with needles the whole surface of the last fully developed leaf. Four different transgenic lines bearing the PHev2.1 promoter region were analyzed. For each line, 7 batches of 3 T1 plants were used. For one batch (T_0), the last fully developed leaf was collected, before any wounding. Five other batches (T_1, T_3, T_6, T_{10} and T_{24}) were submitted to mechanical wounding by pricking with needles the whole limb surface of the last fully developed leaf, then the wounded leaves were collected respectively 1, 3, 6, 10 and 24 hours after wounding. For the last batch ($T_{10}S$), the first and third leaves were wounded, then the intermediary unwounded leaf was collected 10 hours after wounding, in order to test for systemic induction in response to wounding.

The results are shown in Figure 9: for each time point, the GUS activity value represented is the average value from 4 different

transgenic lines, each line being represented by the average value from 3 T1 progenies.

All four lines bearing the PHev2.1 promoter region displayed a significant overexpression of the *uidA* gene in response to wounding, with maximum amplitude observed 10 hours after wounding (amplification factor 1.4 to 1.6). This induction was statistically highly significant ($P<0.01$) as evaluated by Fisher test (LSD).No significant change was observed for the plants bearing the CaMV 35S promoter. The level of activity of the wound-stimulated PHev2.1 promoter is thus very close (80%) to the level displayed by the 35S promoter in similar conditions. Moreover, the PHev2.1 promoter appeared to respond also systemically to wounding. The amplification factor measured on intact leaves framed by wounded leaves 10 hours after wounding was about 1.2 in average.

For the only two lines expressing the *uidA* gene driven by the short promoter region PHev1.1, no modification of the GUS activity in response to wounding could be measured, the expression level being two low.

Inoculation by pathogens:

Six transgenic lines, represented by 3 batches of 3 T1 progenies, were analyzed. For each line, one batch was inoculated with spores (100 000 per ml, in 0.5% gelatin) from the fungus *Magnaporthe grisea* strain TH16 which generates pyriculariose symptoms of medium severity on the rice variety Nipponbare. One full leaf was collected for analysis 4 days after inoculation (I_4). The two other batches were sprayed with 0.5% gelatin only and one full leaf per plant was collected for analysis, either immediately (C_0) or 4 days after treatment (C_4).

The results of fluorimetrical analysis are shown in Figure 10; for each event, the GUS activity value represented is the average value from 6 different transgenic lines, each line being represented by the average value from 3 T1 progenies.

These results show that the inoculation with *Magnaporthe grisea* (strain TH16), agent of the rice pyriculariose, triggers a significant activation of the PHev2.1 promoter. This was observed

for all 6 transgenic lines tested, with amplification factors comprised between 1.2 and 1.4. This induction was statistically highly significant ($P<0.01$) as evaluated by Fisher test (LSD).

EXAMPLE 4 : FUNCTIONAL ANALYSIS IN *ARABIDOPSIS*

Arabidopsis thaliana (ecotype Col0) was transformed *in planta* as described by Clough and Bent (1998). Seeds were collected and stored at 4°C, at least for one week, for vernalisation. Selection of the transgenic events was performed as follows: seed were sterilized for 20 min in a mixture of 30% sodium hypochlorite, absolute ethanol and water (1/4/3; v/v/v), rinsed three times with absolute ethanol and dried for 2 hours. They were then sown and grown *in vitro* on MS/2 medium supplemented with 30 $\mu g.ml^{-1}$ hygromycin. The hygromycin resistant seedlings (transformed events of first generation, or T0) were transferred to the green house after development of the first leaves. Transformed lines of second generation (T1) were similarly selected on hygromycin.

Arabidopsis plantlets bearing the *uidA* gene driven by the PHev2.1 promoter sequence were grown *in vitro* on medium containing hygromycin then tested histochemically for GUS activity. Blue coloration revealing GUS activity was observed in about 65% (17/26) of the hygromycin-resistant lines, in all parts of the plants, both in T0 plants and their hygromycin-selected T1 progenies, demonstrating that the promoter sequence PHev2.1 can be functional in such heterologous system. For the others (35%), no blue coloration was observed.

CLAIMS

1) The use of a polynucleotide selected among:

(a) a polynucleotide having a sequence including nucleotides -254 to -1 of the sequence of Figure 3 (nucleotides 1-254 of SEQ ID NO: 1);

(b) a polynucleotide having a sequence including nucleotides -448 to -1 of the sequence represented of Figure 4 (nucleotides 1-448 of SEQ ID NO: 2);

(c) a polynucleotide having a sequence including nucleotides -1776 to -1 of the sequence of Figure 5, (nucleotides 1-1776 of SEQ ID NO: 3);

(d) a polynucleotide having a sequence including nucleotides -1044 to -1 of the sequence of Figure 6, (nucleotides 1-1044 of SEQ ID NO: 4);

(e) a polynucleotide having a sequence including nucleotides -919 to -1 of the sequence of Figure 7, (nucleotides 1-919 of SEQ ID NO: 5;

(f) a polynucleotide consisting of a fragment of at least 254 bp from the 3'end of a polynucleotide (b) to (e);

(g) a polynucleotide having at least 90% identity with a polynucleotide (a) to (f) and having a promoter function in a plant cell;

as a promoter for driving the expression of an heterologous gene of interest in a plant or a plant cell.

2) The use of claim 1, wherein said polynucleotide is selected among

- a polynucleotide (c) as defined in claim 1;
- a polynucleotide (g) having least 90% identity with said polynucleotide (c) and is used as an inducible promoter regulated by wounds and pathogen infection.

3) A recombinant expression cassette comprising a promoter consisting of a polynucleotide (a) to (g) as defined in any of

claims 1 or 2, and a heterologous polynucleotide placed under transcriptional control of said promoter.

4) A recombinant expression vectors comprising an expression cassette of claim 3.

5) A transformed cell comprising an expression cassette of claim 3.

6) A transgenic plant comprising an expression cassette of claim 3.

7) A transgenic plant of claim 6, wherein said plant is a dicotyledonous.

8) A transgenic plant of claim 7, wherein said plant is *Hevea brasiliensis*.

9) A transgenic plant of claim 6, wherein said plant is a monocotyledonous.

ABSTRACT

PROMOTER SEQUENCES FROM *HEVEA BRASILIENSIS* HEVEIN GENES

The present invention relates to the isolation of DNA sequences from the *Hevea brasiliensis* containing the promoter and regulatory region of hevein genes, and to demonstrate their functionality in *Hevea* as well as in heterologous systems. The present invention also provides a recombinant expression cassette comprising the hevein promoter and a heterologous polynucleotide placed under transcriptional control of said promoter. The present invention further provides recombinant expression vectors comprising an expression cassette of the invention, introduced into host cells and plants to produce transformed cells and transgenic plants. For that purpose, chimaeric gene constructs containing hevein 5' flanking DNA linked to the *uidA* reporter gene was prepared and introduced into *Hevea* callus, and for some of the constructs, in model plants (rice or *Arabidopsis*). Functional analysis conducted by histochemical analysis and by measuring the β-glucuronidase enzyme activity, in various contexts revealed the functionality of the hevein promoter in transformed cells and transgenic plants. The promoter sequences of the hevein genes also acts as an inducible promoter regulated by wounds and pathogen infection.

Primer name and position	Primer sequence
T3	5' ATTAACCCTCACTAAAGGGA 3' (SEQ ID NO : 6)
T7	5' TAATACGACTCACTATAGGG 3' (SEQ ID NO : 7)
OR1 : Hevein, +128 to +103 (FIG. 5)	5' CCTGCTTGCCGACCACATTGCTCAGC 3' (SEQ ID NO: 8)
SCH-S2: pBlueScript SK	5' AACAGCTATGACCATGATTAC 3' (SEQ ID NO: 9)
SCH-R2: Hevein +48 to +27 (FIG. 5)	5' TCAGATCTCCCATTTCTTCCCAATTCTTG 3' (SEQ ID NO: 10) *Bgl II*

FIGURE 1A. Oligonucleotides used in polymerase chain reaction (PCR) on sequencing, for the isolation of the hevein gene promoter region PHev1.1, PHev1.2, PHev2.1, PHev2.2. Restriction enzyme site is underlined. OR1 is hevein-specific primer designed from the cDNA coding sequences (GeneBank M36986), close to the translation initiation codon. The position is numbered from the transcription start site.

Primer name and position	Primer sequence
GSP1 hevein: +174 to +145 (FIG. 7)	5' CTGGCTACAACATAGGTTATTGGGGCAGAG 3' (SEQ ID NO: 11)
GSP2 hevein: +185 to +159 (FIG. 7)	5' GTGGAGCCACACCACCCCCACTGGCTA 3' (SEQ ID NO: 12)
GSP3 hevein: +205 to +176 (FIG. 7)	5' CAGGTGAACAATATTCATCAGTGGAGCCAC 3' (SEQ ID NO: 13)
AP1 Genome Walker Adaptor (CLONTECH)	5' GTAATACGACTCACTATAGGGC 3' (SEQ ID NO: 14)
AP2 Genome Walker Adaptor (CLONTECH)	5' ACTATAGGGCACGCGTGGT 3' (SEQ ID NO: 15)
Hev PF: +55 to +72 (FIG. 7)	*Xba I* 5' GGTCTAGACCCATTTCTTCCCAATTC 3' (SEQ ID NO: 16)
Hev P1: -275 to –258 (FIG. 7)	*Hind III* 5' GGAAGCTTCCTGGCCCTATGCTCTAT 3' (SEQ ID NO: 17)
Hev P2: -389 to –372 (FIG. 7)	*Hind III* 5' GGAAGCTTCGAGTTAACCCTTGCGTT 3' (SEQ ID NO: 18)
Hev P3: -687 to --670 (FIG. 7)	*Hind III* 5' GGAAGCTTGCCCTCTTGGTTGTTGCC 3' (SEQ ID NO: 19)
Hev P4: -919 to -903 (FIG. 7)	*Hind III* 5' GGAAGCTTCGACGGCCCGGGCTGGT 3' (SEQ ID NO: 20)
M13 Forward	5' GTAAAACGACGGCCAG 3' (SEQ ID NO: 21)
M13 Reverse	5' CAGGAAACAGCTATGAC 3' (SEQ ID NO: 22)
SMART III (CLONTECH)	5' AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG 3' (SEQ ID NO : 23)
CDS III/3' PCR Primer (CLONTECH)	5' ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}N_{-1}N$ 3' (SEQ ID NO : 24)
5'PCR Primer (CLONTECH)	5' AAGCAGTGGTATCAACGCAGAGT 3' (SEQ ID NO : 25)
S1 (Gus Fusion Junction)	5' GATTTCACGGGTTGGGGTTTCT 3' (SEQ ID NO: 26)

FIGURE 1B. Oligonucleotides used in the polymerase chain reaction (PCR) or sequencing, for the isolation of the hevein gene promoter region HevP. Restriction enzyme sites are underlined. GSP1, GSP2, GSP3 are hevein-specific primers designed from the cDNA coding sequences Their position is numbered from the transcription start site.

Gene name	**Sequence homology group**	**Cloning I** (full length gene)	**Cloning II** (isolated upstream region)	**Cloning III** (in binary vector)	**/ insert size**
Hev1.1 Hev1.2	Group I	pBS-Hev1.1 pBS-Hev1.2	pGEMT-PHev1.1 pGEMT-PHev1.2	pCambia-PHev1.1	/ 303 bp
Hev2.1 Hev2.2	Group II	pBS-Hev2.1 pBS-Hev2.1	pGEMT-PHev2.1 pGEMT-PHev2.1	pCambia-PHev2.1	/ 1824 bp
Hev2.3 (HevP)	Group II	-	PCR 2.1 TOPO-HevP	pPGTV-KAN-1 pPGTV-KAN-2 pPGTV-KAN-3 pPGTV-KAN-4	/ 323 bp / 436 bp / 734 bp / 966 bp

FIGURE 2. Classification of the hevein genes and their subclones.
Cloning I: genomic sequences including the full transcribed region and 5' upstream sequence of hevein genes, cloned in the pBlueScript phagemid from lambda Zap II (Stratagene).
Cloning II: Promoter regions isolated by PCR. For Hev1.1, Hev1.2, Hev2.1 and Hev2.2, subcloning from the PBS-Hev clones in the pGEMT-easy vector (Promega). For Hev2.3 (HevP), direct cloning by adaptor-anchored PCR in the pCR2.1 TOPO vector (Invitrogen, USA).
Cloning III: Subcloning in a binary vector for transformation. For Hev2.1 and Hev2.2, isolated promoter region cloned between *Hind* III and *Bgl* II sites of the pCambia 1381Z vector (cambia). For Hev2.3, deleted fragments of the isolated promoter region cloned between the *Xba* I and *Hind* III sites of the pPGTV-KAN vector (Becker et al., 1992).

```
-254                                                          AACT
-250  TTATATTATG TGATGTTTTC CCCTTTTAAT TAACTTTATA TGGATTTTTT
-200  TTTTCAAATG CCACCGCTCA ATTCACATTG CAACTTGGCG GTGGCACACA
-150  ATGGCCGCTT TTGTTGACCA AGCTGATTTC TTCTCATGGC CATTTCCCCA
-100  CATGCTTTCT TATATATATT ATTTCTTTTT ACATCCCAAG ATAAACACCT
 -50  TAGCCACATA TCTCTCTGCT ATAAATAAAG CCAAGTGAGC TTAGCTCATC
  +1  ATCATATAAT TTGCAAACCA GAAATTCAAG AATTGGGAAG AAATGGGAAG
 +51  AGTTATGAAT ATATTTATGG TTGTTTTATT ATGTTTAACA GGTGTTGCAA
+101  TTGCTGAGCA ATGTGGTTGG CAAGCAGGTG GCAAGCTCTG CCCCAATAAC
```

FIGURE 3. Nucleotide sequence of the hevein promoter region PHev1.1. The transcription start site (A) and translation initiation codon (ATG) are in bold letters.

```
-448     TTTTTGCA TGCATGTAAT TTTCAACTCA TTTATATATT TATTGTGAAA
-400  TTTTTATTAT ATTATATATA TATATATATA TATATATATA TATATATATA
-350  TATATATATA AATCAGAATT TAAGAGTAAA TGTATATTTT TTTTTGGATA
-300  AATAAAATTT GAAATTTTGT TGCATCAAAT ATCATATTTG AATAATTAAC
-250  TTTATATTAT GTGATGTTTT CCTCTTTTAA TTAACTTTAT ATTGATTTTT
-200  TTTTTAAATG CCACCGCTCA ATTCACATTG CAACTTGGCG GTGGCACACA
-150  ATGGCCACTT TTGTTGACCA AGCTGATTTC TTCTCATGGC CATTTCCCCA
-100  CATGCTTTCT TATATGTATT ATTTCTTTTT ACATCCCAAG ATAAACACCT
 -50  TAGCCACATA TCTCTCTGCT ATAAATAAAG CCAAGTGAGC TTAGCTCATC
  +1  ATCATATCAT TTGCAAACCA GAAAATCAAG AATTGGGAAG AAATGGGAAG
 +51  AGTTATGAAT ATATGTATGG TTGTATTATT ATGTTTAACA GGTGTTGCAA
+101  TTGCTGAGCA ATGTGGTAGG CAAGCAGGTG GCAAGCTCTG CCCCAATAAC
```

FIGURE 4. Nucleotide sequence of the hevein promoter region PHev1.2. The transcription start site (A) and translation initiation codon (ATG) are in bold letters.

```
-1776                                CTTGTT TGCACATGAT GCGTTCAGGT
-1750  GACCAATACT GGAACAGGAG ATAATGTGAC GGTGAAAATT GTTGATCAGT
-1700  GCAGCAATGG AGGTTTGGAC TTAGACGAAG GTGCTTTCCC GCAGATAGAC
-1650  ACCGATGGAA AAGGCTATGC TCAAGGCTAC CTTATTGGGA ACTACCAATT
-1600  TGTGGATTGT GGTGATTGAA TTAACTAATA AGCAACTGAA TGTTAATTTC
-1550  CAGAATAAGA AAACTTGCTG ATTGTAATCT CAAGTTCTAG AGTGAAAATA
-1500  AAGATAATTA TATAAAATAT ATGGAAATTA TTATCCTAGA GGAAATTTTA
-1450  TTTTTTTTTA AATTAATAAA ATTTTTGTAA TTAAAAATTT TACGAAAAAA
-1400  AATCTAATAA AATAAATTTA TGTAAAATTA CTTTATTTTT TATAATAAAA
-1350  TAATTACATT ATGTATGAAA CTAAGTAATC ATAGAATATA TATATATATT
-1300  ATTTAGTTTA TGTGTCAAAT ATAATAGATT AATATTTTCT TTATTATTTT
-1250  TCAAAATAAT TTTCATGTCA ACCCAATTAA ATAAATATCC AACTAATTTT
-1200  TTTTTTAAAT ATTTTTATTT CACAGAGAAT AATTTGTATA TAAAAAATAA
-1150  TTTTCATAAA AATATTTTTT ATTATTTAAT TTTAACATTA ATTAATGGTA
-1100  CGTGTTCATA TTATATATGA ATAATATTTT TATATTTTAA TAAAATTATC
-1050  AAAGTTGAGA AAATGATTTG CTCTTTTAAG TTCTCTCTTA AAAAAGAAAG
-1000  TCATTTTTCT TAAAAATAAT TTAATTTCTC TTTGACTAAA ATATTTTTTG
 -950  TTAATTATTT TTTTAATACT CCAAACACAA AAAATGTGAA AAAAAAAATA
 -900  TTTTCCACGA CACAAACAAA CAGAATTTTA GCCAATCAAT TAGCGCAATT
 -850  TTCAACTCCC CCGCCTCCTA AAGGCTGGAC TGGTGTTGTT CCTGGAGGCT
 -800  GATATCCTAA GCAGGTTTCT GGATTTGCAC TGATTCCATG ATGGTTGAGG
 -750  CAAGAGGGTA TTTCTAATGA GTTTTTATTT AGCCCTCTTG GTTGTTGCCT
 -700  GCCACTGGAA ATCACCATGG AAACATATAT GAAGTCAAAT GACAATTTTT
 -650  ATTTTTTAAA TTTTCTGAGA GTGAGGAAAT GAATAAGAAG AATTTGTTAT
 -600  TTTTCTTTAA AGTCGTGTTA CTTTTACATA ATATATTAAG TCAAATTTAT
 -550  CGACTCAGTG AAAATAATTT ATATTTTATA AATAAGAAAA ATCTTGTTAT
 -500  ATAATTTAAT ATAAATTTTA TATCTTTTTT TTTTCAAGGA AATAAATTTT
 -450  ATATCTTGAT GATAAGATAG AGATAAGATC GAGTTAACCC TTGCGTTAAT
 -400  TGGATGTTTA AATGCTTAAT GCATGGCTAA GGAAATTAAT GTCTAAAATA
 -350  ACAGAAATGA GAAAAATAAA TGAAGGGTGA AAAATAAATA AAACCTGGCC
 -300  CTATGCTCTA TATTGGGGAT GGAGTGGGAG CCACCTAATG TGTCAGTGTT
 -250  CATCTTCGAA CAACGACTCG ATTCAAAGCA CACCCATGAA GCCGCTTCAC
 -200  ATCATCCCTT TGAAACTTTC CACCCTAATC AGCTATCACA CGATCTACTT
 -150  TCCAATCTCA TCAACGCTCC AAATCTCACC ACCATTCAGT CCACTTTCAC
 -100  TTCCTCCTTG TCCTAATCAT CTTTAATCCA TCGGGGTATT ATGGTAATTA
  -50  CATGATCAAG TCTCTCTGCT ATAAATAAAG CCAAGTGAGC TTAGCTCATC
   +1  ATCACATCAT TTGCATACCA GAAAATCAAG AATTGGGAAG AAATGGGAAG
  +51  AGTTATGAAT ACATTTATAG TTGTTTTATT ATGTTTAACA GGTGTTGCAA
 +101  TTGCTGAGCA ATGTGGTCGG CAAGCAGGTG GCAAGCTCTG CCCCAATAAC
```

FIGURE 5. Nucleotide sequence of the hevein promoter region PHev2.1. The transcription start site (A) and translation initiation codon (ATG) are in bold letters.

```
-1044        ATTT TTTTAATATT CCAAACACAA AAAATGTGAA AAAAAAAATA
-1000  TTTTCCACGA CACAAACAAA CAGAATTTTA GCCAATCAAT TAGCGCAATT
 -950  TTCAACTCCC CCGCCTCCTA AAGGCTGGAC TGGTGTTGTT CCTGGAGGCT
 -850  GATATCCTAA GCAGGTTTCT GGATTTGCAC TGATTCCATG ATGGTTGAGG
 -800  CAAGAGGCTA TTTCTAATGA GTTTTTATTT AGCCCTCTGG GTTGTTGCCT
 -750  GCCACTGGAA ATCACCATGG AAACATATAT GAAGTCAAAT GACAATTTTT
 -700  ATTTTTTAAA TTTTCTGAGA GTGAGGAAAT GAATAAGAAG AATTTATTAT
 -650  TTTTCTTTAA AGTCGTGTTA CTTTTACATA ATATATTAAG TCAAATTTAT
 -600  CGACTCAGTG AAAATAATTT ATATTTTATA AATGAGAAAA ATCTTGTTAT
 -550  ATAATTTAAT ATAAATTTTA TATCTTTTTT TTTTTGAAGG AAATAAATTT
 -450  TATATCTTGA TGATAAGATA GAGATAAGAT CGAGTTAACC CTTGCATTAA
 -400  TTGGATGTTT AAATGCTTAA TGCATGGCTA AGGAAATTAA TGTCTAAAAT
 -350  AACAGAAATG AGAAAAATAA ATGAAGGGTG AAAAATAAAT AAAACCTGGC
 -300  CCTATGCTCT ATATTGGGGA TGGAGTGGGA GCCACCTAAT GTGTCAGTGT
 -250  TCATCTTCGA ACAACGACTC GATTCAAAGC ACACCCATGA AGCCGCTTCA
 -200  CATCATCCCT TTGAAACTTT CCACCCTAAT CAATATCACA CGATCTACTT
 -150  TCCAATCTCA TCAACGCTCC AAATCTCACC ACCATTCAGT CCACTTTCAC
 -100  TTCCTCCTTG TCCTAATCAT CTTTAATCCA TCAGGGTATT ATGGTAATTA
  -50  CATGATCAAG TCTCTCTGCT ATAAATAAAG CCAAGTGAGC TTAGCTCATC
   +1  ATCACATCAT TTGCATACCA GAAAATCAAG AATTGGGAAG AAATGGGAAG
  +51  AGTTATGAAT ATATTTATAG TTGTTTTATT ATGTTTAACA GGTGTTGCAA
 +101  TTGCTGAGCA ATGTGGTCGG CAAGCAGGTG GCAAGCTCTG CCCCAATAAC
```

FIGURE 6. Nucleotide sequence of the hevein promoter region PHev2.2. The transcription start site (A) and translation initiation codon (ATG) are in bold letters.

```
-919                                                CGACGGCCC GGGCTGGTAT
-900  TCCAAACACA AAAAATGTGA AAAAAAAAAT ATATTTTCCA CGACACAAAC
-850  AAACAGAATT TTAGCCAATC AATTAGCGCA ATTTTCAACT CCCCCGCTGC
-800  TAAAGGCTGG ACTGGTGTTG TTCCTGGAGG CTGATATCCT AAGCAGGTTT
-750  CTGGATTTGC ACTGATTCCA TGATGGTTGA GGCAAGAGGG TATTCCTAAT
-700  GAGTTTTTAT TTAGCCCTCT TGGTTGTTGC CTGCCACTGG AAATCACCAT
-650  GGAAACATAT ATGAAGTCAA ATGACAATTT TTATTTTTTA AATTTTCTGA
-600  GAGTGAGGAA ATGAATAAGA AGAATTTGTT ATTTTTCTTT AAAGTCGTGT
-550  TACTTTTACA TAATATATTA AGTCAAATTT GTCGACTCAG TGAAAATAAT
-500  TTATATTTTA TAAATGAGAA AAATCTTGTT ATATAATTTA ATATAAATTT
-450  TATATCTTTT TTTTTTGAAG GAAATAAATT TTATATCTTG ATGATAAGAC
-400  AGAGATAAGA TCGAGTTAAC CCTTGCGTTA ATTGGATGTT TAAATGCTTA
-350  ATGCATGGCT AAGGAAATTA ATGTCTAAAA TAACAGAAAT GAGAAAAATA
-300  AATGAAGGGT GAAAAATAAA TAAAACCTGG CCCTATGCTC TATATTGGGG
-250  ATGGAGTGGG AGCCACCTAA TGTGTCAGTG TTCATCTTCG AACAACGACT
-200  CGATTCAAAG CACACCCATG AAGCCGCTTC ACATCATCCC TTTGAAACTT
-150  TCCAATCTCA TCAACGCTCC AAATCTCACC ACCATTCAGT CCACTTTCAC
-100  TTCCTCCTTG TCCTAATCAT CTTTAATCCA TCAGGGTATT ATGGTAATTA
 -50  CATGATCAAG TCTCTCTGCT ATAAATAAAG CCAAGTGAGC TTAGCTCATC
  +1  ATCACATCAT TTGCATACCA GAAAATCAAG AATTGGGAAG AAATGGGAAG
 +51  AGTTATGAAT ATATTTATAG TTGTTTTATT ATGTTTAACA GGTGTTGCAA
+101  TTGCTGAGCA ATGTGGTCGG CAAGCAGGTG GCAAGCTCTG CCCCAATAAC
+151  CTATGTTGTA GCCAGTGGGG GTGGTGTGGC TCCACTGATG AATATTGTTC
+201  ACCTG
```

FIGURE 7. Nucleotide sequence of the PHev2.3 (HevP) hevein promoter region, isolated by nested PCR. The transcription start site (A) and translation initiation codon (ATG) are in bold letters.

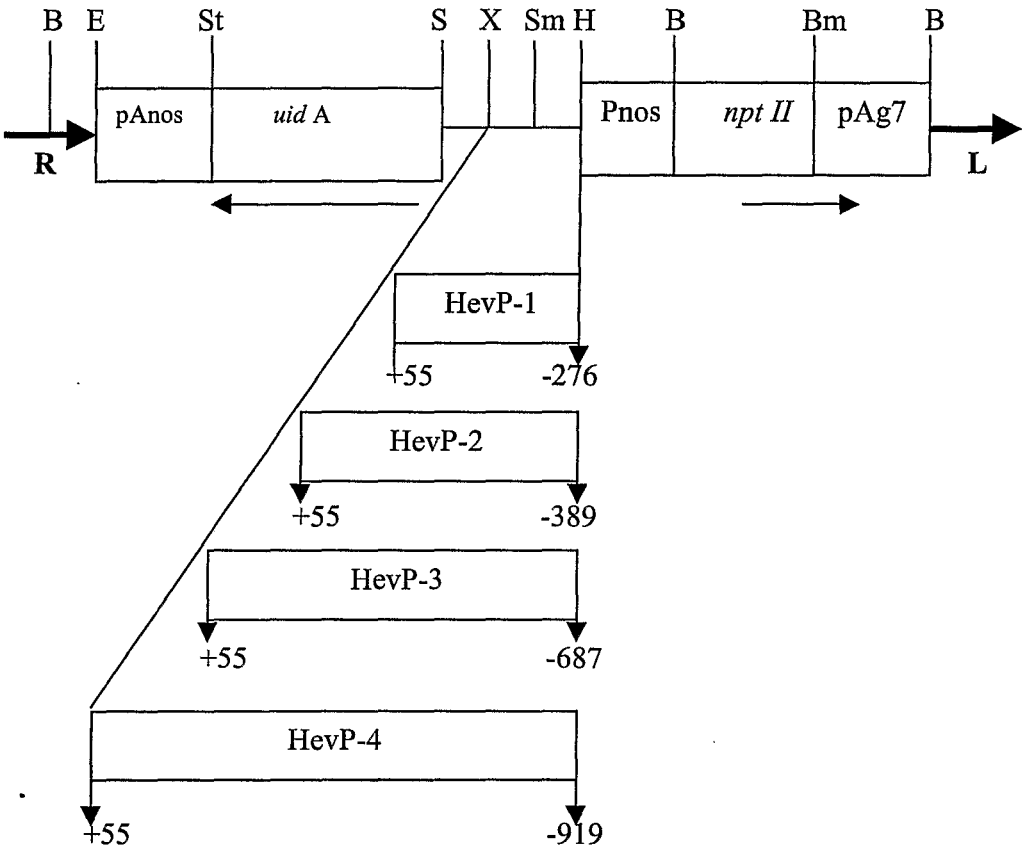

FIGURE 8. Schematic diagram of the PCR-generated nested deletion fragments obtained from the hevein promoter region HevP (Phev2.3) and cloned in pPGTV-KAN. The promoter sequences were joined to the *uidA* reporter gene. The 5' and 3' end points of the promoter sequences are numbered from the transcription start site of the hevein gene. pPGTV-KAN-1 (HevP-1 : -275 to +55) ; pPGTV-KAN-2 (HevP-2: -389 to +55) ; pPGTV-KAN-3 (HevP-3: -687 to +55) and pPGTV-KAN-4 (HevP- : -919 to +55). The binary vector pPGTV-KAN contains unique cloning sites upstream of the *uidA* gene which allows the insertion of promoter fragments. The T-DNA nopaline synthase (pAnos) and gene 7 (pAg7) poly (A) signals follow the *uidA* gene and the selectable marker (*nptII*) genes respectively. Arrows indicate direction of transcription ; R, right T-DNA border ; L, left T-DNA border. Abbreviations :
St=*Sst* I, E= *Eco* RI, Sm=*Sma* I, X=*Xba* I, S=*Sal* I, H=*Hind* III, B=*Bgl* II, Bm=*Bam* HI, *npt II*= neomycin phosphotransferase II gene.

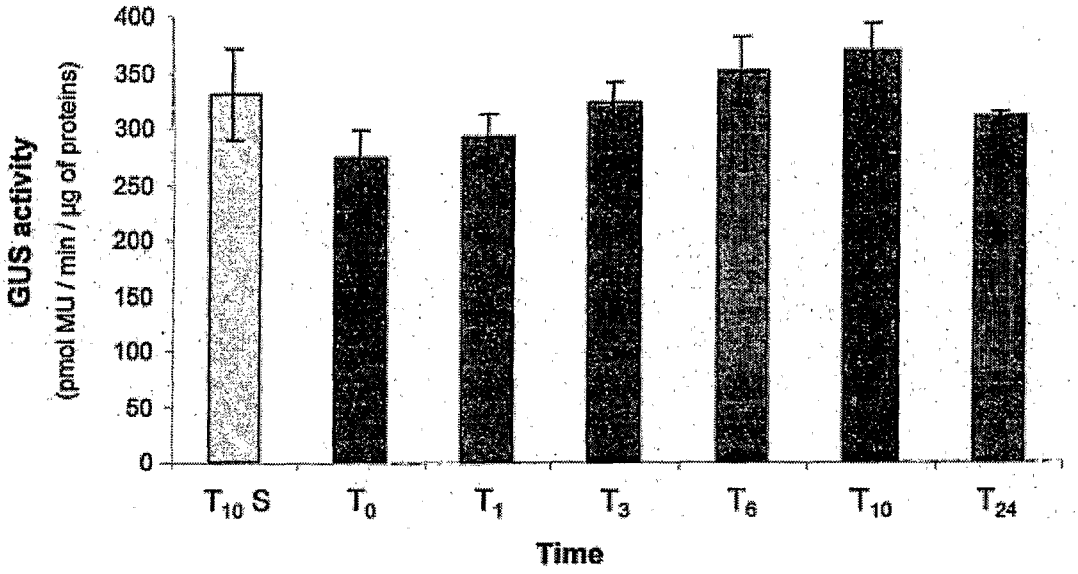

FIGURE 9. Fluorometric analysis of the GUS activity in transgenic rice plants (T1) carrying a single copy of the *uidA* gene driven by PHev2.1, in response to mechanical wounding. For each time point, the GUS activity value represented is the average value from 4 different transgenic lines, each line being represented by the average value from 3 T1 progenies. Wounding was performed by pricking leaves with needles. Wounded leaves were collected 1, 3, 6, 10 and 24 hours after wounding (T1, T3, T6, T10, T24 respectively). T0 : unwounded plants. T10S : systemic response of unwounded intermediary leaves located between 2 wounded leaves and analysed 10 hours after wounding. The statistical significance was evaluated by Fisher test ($P<0.01$).

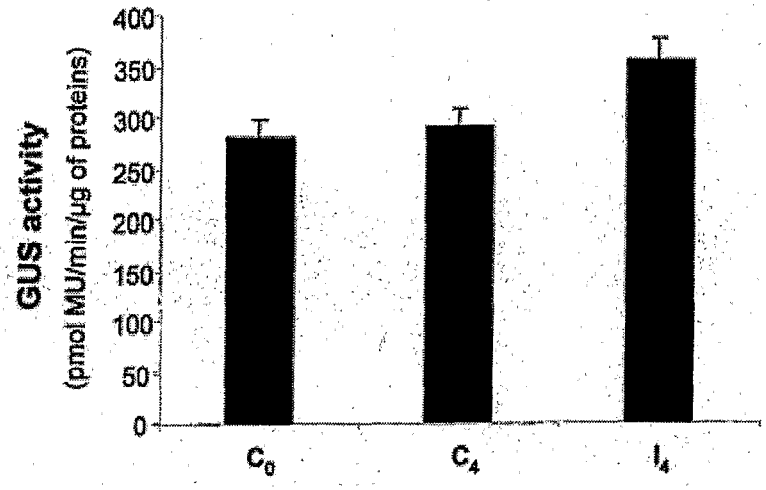

FIGURE 10. Fluorometric analysis of the GUS activity in transgenic rice plants (T1) carrying a single copy of the *uidA* gene driven by PHev2.1, in response to fungal infection. For each event, the GUS acivity value presented is the average from 6 different transgenic lines, each being represented by the average value from 3 T1 progenies. Control plants were sprayed with 0.5% gelatin and anlaysed at the time points 0 (C_0) or 4 days after treatment (C_4) ; I_4 : plants innoculated with spores of *Magnaporthe grisea* (TH16) in 0.5% gelatin and analysed 4 days after innoculation. The statistical significance was evaluated by Fisher test ($P<0.01$).